UNIVERSITI PUTRA MALAYSIA

CRYOPRESERVATION OF PROTOCORM-LIKE BODIES OF ORCHID HYBRID *DENDROBIUM* KASEM BOONCHOO WHITE BY VITRIFICATION

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FP 2000 14

CRYOPRESERVATION OF PROTOCORM-LIKE BODIES OF ORCHID HYBRID *DENDROBIUM* KASEM BOONCHOO WHITE BY VITRIFICATION

Thesis Submitted in Fulfilment of the Requirements for the Degree of Master of Agricultural Science in the Faculty of Agriculture Universiti Putra Malaysia

August 2000

Dedicated to:

My parents:

Wilson Sipen Sawap & Ranyod Sigeng

My siblings:

Perisen Kichiak & family, Peter Aat & family, Patrick Sungeh & family,
Prentis Kenneth, Pirin Decem and Polinomi

My relatives and friends

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Agricultural Science.

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By

PHILIP ANAK SIPEN

August 2000

Chairman : Associate Professor Saleh Bin Kadzimin, Ph. D.

Faculty : Agriculture

The present study was conducted to develop a protocol for long-term preservation of orchid germplasm. Protocorm-like bodies (PLBs) of orchid hybrid *Dendrobium* Kasem Boonchoo White were subjected to different treatments of preculture, cryoprotection and dehydration procedures of vitrification technique.

The PLBs were precultured on Vacin and Went medium supplemented with 0, 0.06, 0.1, 0.3, 0.5 and 0.7 M sucrose for 3 days. Preculturing with 0.06 to 0.5 M sucrose gave 3.7 to 23.3% viability after cryopreservation. No viable PLBs were found from 0 and 0.7 M sucrose treatments. None of the PLBs survived cryopreservation in all the sucrose concentrations tested.

The PLBs were precultured with 0.3 M sucrose for 0, 1, 3, 5, 7 and 9 days. Viability ranged from 11.1 to 26.3% after cryopreservation from 1 to 9 days treatments.

No viability was recorded after cryopreservation from treatment without sucrose preculture. There was no survival recorded in all treatments after cryopreservation.

When precultured PLBs were cryoprotected with different loading solutions (LS1, LS2, LS3, LS4, LS5 and LS6) for 15 min, all treatments gave 7.4 to 34.4% viability after cryopreservation. All treatments gave 3.7 to 11.1% survival after cryopreservation except LS1 and LS2 treatments.

PLBs were cryoprotected with LS5 for 0, 5, 10, 15, 20, 25 and 30 min. Viability of 7.4 to 36.8% were recorded after cryopreservation when cryoprotected for 5 to 30 min. Without cryoprotection, viability was zero after cryopreservation. The survival rates of 11.1 to 30.3% were obtained after cryopreservation from 5 to 25 min treatments. Cryoprotection for 30 min and without cryoprotection did not give any survival at all after cryopreservation.

Cryoprotected PLBs were dehydrated with different vitrification solutions (VS1, VS2, VS3, VS4 and VS5) for 10 min. All vitrification solutions gave 11.1 to 39.2% viability after cryopreservation. Survival of 7.4 to 26.3% after cryopreservation were recorded in all treatments except VS1.

Cryoprotected PLBs were dehydrated with VS3 for 0, 5, 10, 15, 20, 25 and 30 min. Viability range of 20.1 to 53.2% after cryopreservation were recorded when dehydrated for 5 to 30 min. No viability was noted after cryopreservation without dehydration. Survival after cryopreservation was 3.7 to 48.5% from 5 to 30 min

treatments. The highest survival (48.5%) after cryopreservation was obtained from 30 min. None of the cryopreserved PLBs survived after cryopreservation without dehydration.

The highest rate of survival of cryopreserved PLBs achieved with the vitrification technique in the study was 48.5%. Thus, vitrification technique appears promising for the cryopreservation of orchid PLBs, proposing a protocol for long-term preservation of orchid germplasm.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains Pertanian.

PENGKRIOAWETAN PROTOKOM HIBRID ORKID *DENDROBIUM* KASEM BOONCHOO WHITE MELALUI VITRIFIKASI

Oleh

PHILIP ANAK SIPEN

Ogos 2000

Pengerusi Penyelia : Prof. Madya Saleh Bin Kadzimin, Ph.D.

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Kajian ini telah dijalankan untuk mencipta satu protokol bagi pengekalan jangka panjang janaplasma orkid. Protokom (PLBs) hibrid orkid *Dendrobium* Kasem Boonchoo White telah diberi rawatan prakultur, perlindungankrio dan pengdehidratan yang berbeza.

PLBs telah diprakultur di atas medium Vacin dan Went yang mengandungi 0, 0.06, 0.1, 0.3, 0.5 dan 0.7 M sukrosa selama 3 hari. Prakulturan dengan 0.06 hingga 0.5 M sukrosa telah memberikan 3.7 hingga 23.3% viabiliti selepas pengkrioawetan. Tiada viabiliti didapati selepas pengkrioawetan dari rawatan 0 dan 0.7 M sukrosa. Tiada kemandirian PLBs direkodkan selepas pengkrioawetan di dalam semua rawatan.

PLBs telah diprakultur pada 0.3 M sukrosa selama 0, 1, 3, 5, 7 dan 9 hari. Kadar viabiliti 11.1 hingga 26.3% selepas pengkrioawetan telah direkodkan dalam prakultur selama 1 hingga 9 hari. Tiada viabiliti selepas pengkrioawetan dalam rawatan tanpa prakultur dengan sukrosa. Tiada kemandirian direkodkan dalam semua rawatan selepas pengkrioawetan.

Apabila PLBs yang telah diprakultur diberi perlindungankrio dengan larutan loading (LS1, LS2, LS3, LS4, LS5 dan LS6) selama 15 min, kesemua rawatan memberikan 7.4 hingga 34.4% viabiliti selepas pengkrioawetan. Kesemua rawatan memberikan 3.7 hingga 11.1% kemandirian selepas pengkrioawetan kecuali rawatan LS1 dan LS2.

PLBs diberi perlindungankrio dengan LS5 selama 0, 5, 10, 15, 20, 25 dan 30 min. Kadar viabiliti PLBs adalah 7.4 hingga 36.8% apabila diberi perlindungankrio selama 5 hingga 30 min Tanpa perlindungankrio, tiada viabiliti selepas pengkrioawetan direkodkan. Kadar kemandirian PLBs yang telah dikrioawetkan sebanyak 11.1 hingga 30.3% telah diperolehi dari rawatan 5 hingga 25 min. Rawatan 30 min dan tanpa perlindungankrio menunjukkan tiada kemandirian selepas pengkrioawetan.

PLBs yang telah diberi perlindungankrio didehidratkan dengan larutan-larutan vitrifikasi yang berlainan (VS1, VS2, VS3, VS4 dan VS5) selama 10 min. Kesemua larutan vitrifikasi telah memberikan kadar viabiliti 11.1 hingga 39.2% selepas pengkrioawetan. Kadar kemandirian 7.4 hingga 26.3% selepas pengkrioawetan telah direkodkan dari kesemua rawatan.

PLBs yang telah diberi perlindungankrio kemudiannya didehidratkan dengan VS3 selama 0, 5, 10, 15, 20, 25 dan 30 min. Kadar viabiliti 20.1 hingga 53.2% selepas pengkrioawetan telah direkodkan selepas didehidratkan selama 5 hingga 30 min. Tiada viabiliti dari rawatan tanpa dehidrasi selepas pengkrioawetan. Kemandirian selepas pengkrioawetan adalah sebanyak 3.7 hingga 48.5% dari rawatan 5 hingga 30 min. Kadar kemandirian tertinggi (48.5%) selepas pengkrioawetan telah diperolehi dari 30 min. Tiada PLBs yang mandir selepas pengkrioawetan tanpa dehidrasi dengan VS3.

Kadar kemandirian tertinggi PLBs yang terus hidup selepas pengkrioawetan dengan menggunakan teknik vitrifikasi ialah 48.5%. Dengan itu, teknik vitrifikasi berkenaan adalah berpotensi untuk digunakan sebagai kaedah pengawetan PLBs orkid, dan seterusnya menjadi satu protokol bagi pengekalan jangka panjang janaplasma orkid.

ACKNOWLEDGEMENTS

I am grateful to the Lord Almighty for His blessings and guide me through always

I would like to express my sincere appreciation and heartfelt gratitude to my supervisor, Assoc. Prof. Dr. Saleh b. Kadzimin for his wise, valuable counsel and suggestions, with constant support and encouragement that made this study possible. His understanding and patience in the study were most comforting and necessary.

Thanks are also due to my co-supervisors, Prof. Dr. Noraini bte. Abd. Samad and Assoc. Prof. Dr. Hor Yue Luan for their supportive advice, attention and constructive criticism in this study.

The author also wishes to thank En. Abd. Rahman b. Sidam, Haryati bte. Jamhari, Salimah bte. Ali, Asnita bte. Abu Harirah, Dahroyana Nor and Elixon Sunian of Tissue Culture Laboratory for their kind support. I am grateful to the staff of the Electron Microscope Unit of UPM, Cik Azilah Abd. Jalil, Puan Suleka Madhavan, En. Ho Oi Kuan and Puan Faridah Akmal for their assistance in electron microscopy work.

Finally but not least, I thank my friends Nurasykin Abdullah, Florence Ginibun, Wong Lay Yieng, Sam Yen Yen, Adrain Ling, Norsheda Abdullah, Mohana Anita, Franklin Ragai, Felix Angkau, Lee Aji, Ismail b. Ibrahim, Hendry Joseph, Mohd. Humayun Kabir and Make Jiwan for their kind supports.

I certify that an Examination Committee met on 14th August 2000 to conduct the final examination of Philip Anak Sipen on his Master of Agricultural Science thesis entitled "Cryopreservation of Protocorm-like Bodies of Orchid Hybrid *Dendrobium* Kasem Boonchoo White by Vitrification" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia and was accepted as fulfilment of the requirements for the degree of Master of Agricultural Science.

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DECLARATION

I hereby declare that the thesis is based on my original work except for the quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

PHILIP ANAK SIPEN

Date: 15/9/2000

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
DAS-ELISA	Double antibody sandwich-enzyme linked immunorsobent assay
DMSO	Dimethyl sulfoxide
EG	Ethylene glycol
LN	Liquid nitrogen
MARDI	Malaysian Agricultural Research and Development Institute
MC	Moisture content
min	Minutes
P	After preculture
PL	After preculture and loading
PLV	After preculture, loading and vitrification
PLVC	After preculture, loading, vitrification and cryopreservation
PLBs	Protocorm-like bodies
psi	Pound per square inch
RCBD	Randomised complete block design
SAS	Statistical analytical system
SEM	Scanning electron microscope
VW	Vacin and Went medium, 1949
w/v	Weight-to-volume

CHAPTER I

INTRODUCTION

The family Orchidaceae is the largest of all plant families with a world record of more than 800 genera, at least 25,000 to 30,000 species and many thousands of hybrids. It is a family of great diversity, assuming different life forms such as epiphyte, terrestrial and saprophyte, and producing exquisite flowers with a wide range of colours, sizes and floral forms. Malaysia recorded 110 genera and about 808 species, many of which are epiphytes or terrestrials. Large numbers of these wild species have been routinely utilized in hybridization and Malaysian hybrids are world renown in the orchid industry. Its tremendous floral diversity has made it one of the major cut-flower types in Malaysia contributing a major share in the floriculture industry. However, many of our valuable orchid germplasm are becoming rare and extinct. Therefore, there is an urgent need for the conservation of our still existing orchid germplasm both *in situ* and *ex situ*, particularly for those with high horticultural value.

The *in situ* conservation through establishment of national parks or forest reserves does not ensure the safety of orchid species which exist in those areas. They are exposed to natural disasters, climatic perturbations, pests, pathogens and diseases. The most dangerous threat arises from human who ignore laws preventing the collection of wild orchids. Orchid species and hybrids are also conserved *ex situ* as living collections in orchidariums, botanical gardens, field genebanks, farms, nurseries or glasshouses. In

Malaysia, similar efforts have been stepped up by the Malaysian Agricultural Research and Development Institute (MARDI). Kuala Lumpur Orchid Garden has also been set up which houses various orchid species and hybrids. However, collections are also exposed to similar problems, but to a lesser extent such as natural disasters, attack by pests and pathogens, in addition to high labour costs and technical personnel requirements. These problems as well as heterozygosity in orchids have led conservationists to store orchid materials as an alternative means of conservation.

Storage of orchid seeds provides an alternative conservation measure and has been fairly well documented. However, some orchid seeds are recalcitrant, hence seed bank is not a practical solution to genetic conservation of such species. In addition, fresh seeds need to be harvested to replace the old ones. Pollen and tissue storage has not received much attention.

The tissue culture system of storage offers a good alternative to conventional seed or pollen storage. *In vitro* culture techniques broaden the options available for *ex situ* conservation of orchid germplasm. There are two ways of conserving germplasm *in vitro*: slow growth for slow- to medium-term storage, and cryopreservation for long-term storage. Cryopreservation involves storing plant materials at the temperature of liquid nitrogen (LN) at –196 ^{0}C. In orchids, several attempts have been made to develop protocols for cryopreservation. Cryogenic protocols such as freezing seeds, pollens and protocorm-like bodies (PLBs) of *Vanda*, zygotic protocorms of *Cymbidium*, zygotic embryos of *Bletilla*, protocorms of *Cymbidium* and callus of *Doritaenopsis* have been

reported, utilizing various cryopreservation techniques available. The techniques used varied, resulting in a range of viability.

The present study was undertaken to elucidate the effects of cryopreservation through vitrification technique and to establish a simple, reliable and low-technology protocol for the cryopreservation of orchid cultures. To achieve this, various procedures involved in the vitrification technique need to be optimized. Therefore, the specific objectives of the study are as follows:

1. to determine an optimum concentration and duration of preculturing with sucrose for survival of PLBs in LN.
2. to determine an effective loading solution and exposure time for survival of PLBs in LN.
3. to determine an effective vitrification solution and exposure time for survival of PLBs in LN.